The Herpes Simplex Virus Amplicon: A New Eucaryotic Defective-Virus Cloning–Amplifying Vector

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Summary

We have employed repeat units of herpes simplex virus (HSV) defective genomes to derive a cloning–amplifying vector (amplicon) that can replicate in eucaryotic cells in the presence of standard HSV helper virus. The design of the HSV amplicon system is based on the previous observation that co-transfection of cells with helper virus DNA and seed monomeric repeat units of HSV defective genomes results in the regeneration of concatemeric defective genomes composed of multiple reiterations of the seed repeats. Cotransfection of cells with helper virus DNA and chimeric repeat units containing bacterial plasmid pKC7 DNA resulted in the generation of defective genomes composed of reiterations of the seed HSV–pKC7 repeats. These chimeric defective genomes were packaged into virus particles and could be propagated in virus stocks, with the most enriched passages containing more than 90% chimeric defective genomes. Furthermore, mono-meric chimeric repeat units could be transferred back and forth between bacteria and eucaryotic cells. A derivative vector constructed so as to contain several unique restriction enzyme sites could be potentially employed in the introduction of ad-ditional viral or eucaryotic DNA sequences into eu-caryotic cells.

Introduction

Approximately 10 years ago Jackson et al. (1972) described the construction of hybrid DNA molecules containing linked λdvgal and SV40 DNA sequences, and predicted the potential use of chimeric hybrids in the directed transfer of foreign genes into the heter-ologous eucaryotic cell and bacterial hosts. Recent years have seen the use of SV40-derived packaged vectors in studies of defined sets of foreign DNA sequences, through constructions involving recombi-nant DNA approaches (Ganem et al., 1976; Goff and Berg, 1976, 1979; Hamer et al., 1977, 1979, 1980; Mulligan et al., 1979; Gruss and Khoury, 1981; Mor-iarty et al., 1981; Pavlakis et al., 1981; Sveda and Lai, 1981; Hartman et al., 1982; White et al., 1982). These defective-virus vector systems typically consist of two components: defective viruses containing chi-meric genomes in which the foreign DNA sequences are linked to subsets of viral DNA sequences speci-fying cis replication functions (such as an origin for DNA replication); and adequate helper viruses capa-ble of providing trans replication and packaging func-

tions (including structural components of the virion) needed for the propagation of the chimeric defective genomes.

Because many eucaryotic genes of potential inter-est exceed the size limit of approximately 5 kilobase pairs (kb) that could be accommodated within the SV40 virion, it seemed desirable to develop virus vectors derived from larger DNA-containing animal viruses. Specifically, we have previously suggested that defective genomes of herpes simplex viruses (HSVs) might constitute potentially useful reagents for the cloning and amplifying of foreign genes (Frenkel, 1981). Pertinent to this expectation were several con-siderations. First, the HSV virion can accommodate relatively large (approximately 150 kb) DNA mole-cules. Second, the 150 kb defective-virus DNA mol-ecules that are present in serially passaged virus stocks consist of multiple reiterations of sequences (repeat units) arranged in a head-to-tail tandem array, with repeat unit sizes ranging from 3 to 30 kb (re-viewed by Frenkel, 1981). This feature provides the potential for the amplification of the foreign DNA se-quences within uniform head-to-tail reiterated defec-tive-virus genomes. Third, defective-virus genomes appear to be relatively stable during serial undiluted virus propagation (Locker and Frenkel, 1979a; Fren-kel, 1981; Locker et al., 1982). Finally, full-length defective HSV genomes containing multiple head-to-tail repeat units can be regenerated from individual monomeric repeats, following cotransfection of cells with helper virus DNA. The regenerated concatemers are packaged into structural virions and can be stably propagated in serially passaged virus stocks (Vlazny and Frenkel, 1981; Vlazny et al., 1982). This feature provides a basis for the potential derivation of reiter-ated chimeric defective genomes from seed repeat units that contain foreign DNA sequences linked to HSV cis replication functions.

We have used DNA sequences derived from the bacterial plasmid pKC7 to demonstrate that foreign DNA sequences can be introduced into concatemeric chimeric defective genomes that are efficiently pack-aged and that can be stably propagated in serially passaged virus stocks.

Results

The Choice of Defective-Genome Repeat Units Designed to Serve As the Cloning–Amplifying Vector (Amplicon)

Prior to initiating our studies designed to obtain chi-meric defective genomes containing foreign DNA se-quences, we had to select the defective HSV repeat units that would be used as the vector in such ap-proaches. Of various class I and class II defective HSV genomes present in a number of series that we have studied (reviewed by Frenkel, 1981), we have chosen a minor species present in a series derived by B.

Murray and his coworkers from the Patton strain of HSV1. These defective genomes consist of multiple head-to-tail repeat units in which a stretch of U_L sequences located within map coordinates 0.407–0.429 of standard virus DNA is linked to a small stretch of sequences (less than 0.5 kb) derived from the end of the S component (R. R. Spaete, L. P. Deiss, N. Frenkel and B. Murray, manuscript in preparation; Figure 1). The selected defective genomes possessed two convenient properties. First, they consisted of relatively small repeat units of 3.9 kb. Second, they contained a single Bgl II site that was not present in certain other class II defective genome species analyzed in our laboratory (Locker et al., 1982). It was therefore reasonable to assume that interruptions at the Bgl II site during the construction of chimeric repeat units would

not disrupt any of the cis recognition signals necessary for the propagation of defective HSV genomes.

Regeneration of Concatemeric Defective Genomes from Seeds Corresponding to the 3.9 kb Repeat Units

The first set of experiments with the 3.9 kb Patton repeat units was designed to test the ability of these repeat units to regenerate full-length defective-virus genomes and to serve as amplifying vectors for the generation of concatemeric chimeric defective genomes containing foreign DNA sequences. The experimental design for these studies (Figure 2) generally followed the protocol described by us for the regeneration of full-length defective-virus DNA molecules from defective-genome seed repeats (Vlazny and Frenkel, 1981). Specifically, serially passaged Patton virus DNA was cleaved with the Bgl II enzyme and resultant fragments were electrophoresed in agarose gels. The separated 3.9 kb monomeric repeat was eluted from the gel, and was then employed in two sets of transfections (Tr). The first set included two replicate rabbit skin cell cultures (Tr2 and Tr3) which were cotransfected with a mixture of HSV
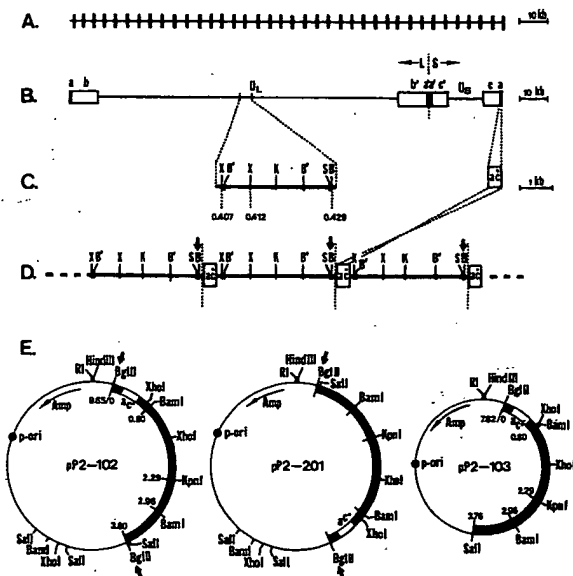

Figure 1. Structures of the HSV1 (Patton) Defective Genomes and Derivative Recombinant Plasmids

(A) Schematic representation of the defective Patton genomes, containing approximately 38 reiterations of 3.9 kb repeat units. The defective genomes terminate at one end with sequences corresponding to the ac terminus of standard virus DNA. (B) Schematic representation of the standard HSV DNA, displaying the arrangement of the unique and inverted repeat sequences of the S and L components (reviewed by Roizman, 1979). (C) Arrangement of the Xho I (X), Bam I (B'), Kpn I (K), Sal I (S) and Bgl II (B) restriction enzyme sites in the U_L segment of standard HSV DNA bounded within the map coordinates shown. ac*: the portion of the S inverted repeat sequences present in the defective genome repeat units. This region is 500 bp maximum size. The asterisk denotes uncertainty in the amount of c sequence present in the 3.9 kb Patton defective-genome repeat units. (D) The arrangement of the U_L and ac* sequences within the Patton defective genomes (R. R. Spaete, L. P. Deiss, N. Frenkel and B. K. Murray, manuscript in preparation). The junctions between adjacent repeats are represented by the dotted vertical lines: the a sequences begin approximately 190 bp from the Bgl II site. Arrows: Bgl II cleavage site used for the introduction of the repeat unit into pKC7. (E) Representation of the pP2-102, pP2-201 and pP2-103 recombinant clones. p-ori: replication origin of the plasmid. Amp: the β-lactamase gene.
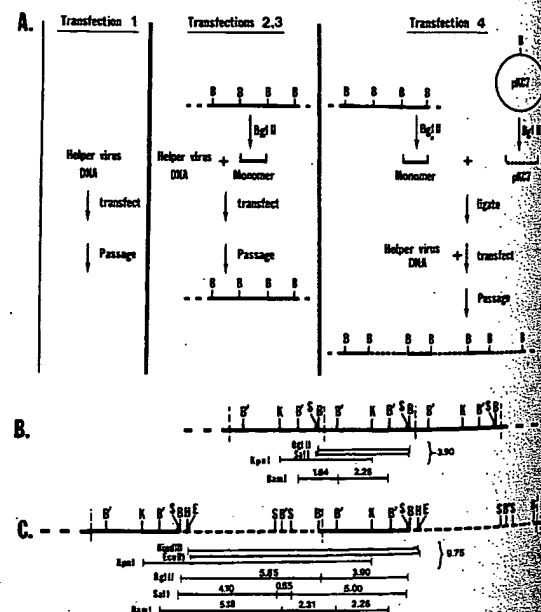

Figure 2. Scheme of the Cotransfection Experiment and the Structure of the Expected Regenerated Defective Genomes.

(A) Transfections 1–4 contained 0.5 µg helper HSV1 (Justin) DNA. In addition, transfections 2 and 3 contained 0.05 µg of the gel-purified Bgl II–digested 3.9 kb repeat units; and transfection 4 contained 0.1 µg of equimolar ligation mixture of the Bgl II–cleaved 3.9 kb repeat and pKC7 DNA. (B) Schematic representation of defective genomes expected to be regenerated in transfections 2 and 3. Thin horizontal lines: fragments expected from digestions with the enzymes shown. Numbers: sizes in kilobase pairs. (C) Schematic representation of chimeric defective genomes expected to be generated in transfection 4. Enzyme abbreviations are as described in Figure 1: E Eco RI, H Hind III.

(Justin) helper virus DNA and the Bgl II–cleaved 3.9 kb repeat. For the second set of transfections, we first ligated Bgl II–linearized (5.8 kb) pKC7 DNA to the cleaved repeat of the Patton defective genomes. The entire ligation mixture was then used to transfect one culture (Tr4) of rabbit skin cells along with helper virus DNA. An additional rabbit skin cell culture (Tr1) was transfected solely with helper virus DNA (without added seed repeat units), to probe for the generation of "endogenous" defective-virus genomes derived from the input helper virus DNA.

Following sequential undiluted propagation of the resultant transfection-derived virus stocks, ^{32}P-labeled DNA was prepared from cells infected with the fifth passages of Tr1 through Tr4. Restriction enzyme analyses of these DNA preparations (Figures 3 and 4) have yielded the following results. First, the serially passaged virus stocks propagated from the control Tr1 (which received helper virus DNA alone) contained "endogenous" defective-virus DNA molecules that appeared to be derived from the S component of standard DNA and thus to correspond to the previously described class I defective HSV genomes (see

legend to Figure 3). Second, passages derived from Tr2 and Tr3 (which received the 3.9 kb seed repeat units along with helper virus DNA) contained major proportions of regenerated defective genomes consisting of repeat units that were indistinguishable from the input 3.9 kb monomers. These virus stocks also contained variable proportions of endogenous class I defective genomes, as well as additional defective genomes most likely arising from deleted 3.9 kb seed DNA molecules that were trimmed within the cells at the Bgl II–cleaved ends. Third, viral DNA from cells infected with progeny virus of Tr4 (which received the HSV–pKC7 ligation mixture) contained a relatively high proportion of defective genomes that consisted of head-to-tail reiterations of the 9.7 kb ligated HSV–pKC7 seed repeats. This conclusion is based on the patterns of single and double restriction enzyme digests of Tr4-derived DNA (Figures 3 and 4), which resulted in the generation of fragments of sizes predicted for such chimeric defective genomes (schematically shown in Figure 2). Furthermore, in an additional experiment shown in Figure 4, we have prepared full-length Hpa I-resistant virus DNA from cells
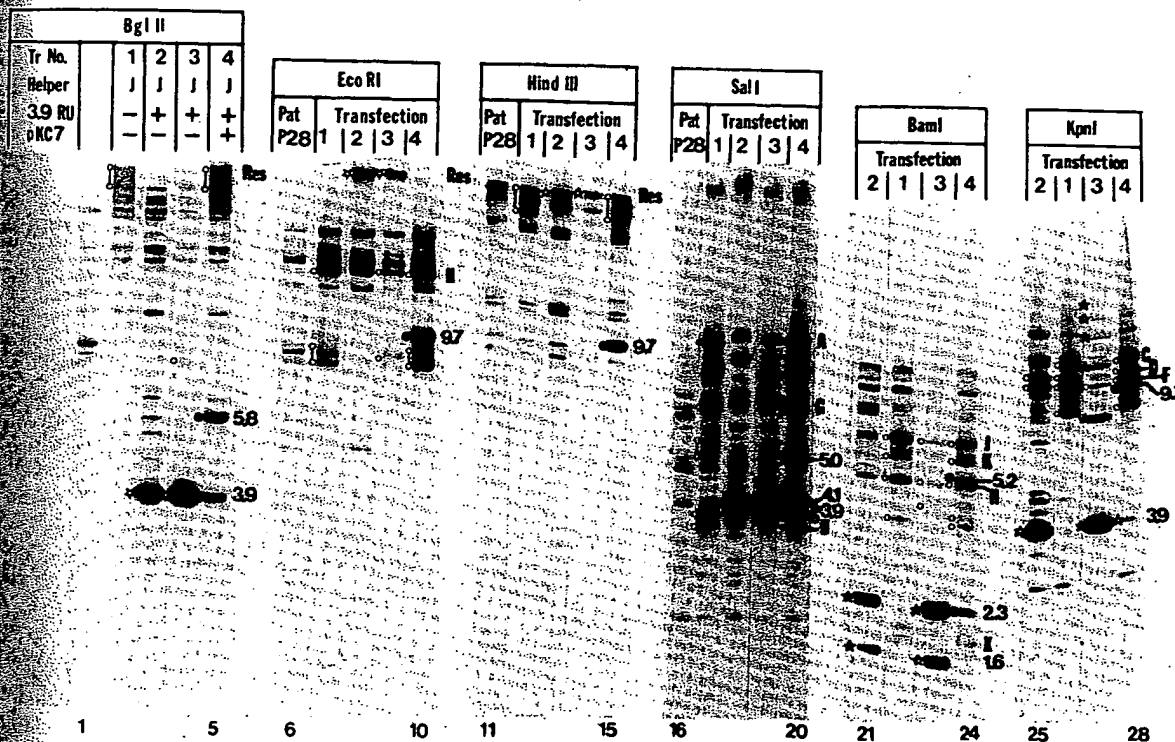

Figure 3. Restriction Enzyme Analyses of ^{32}P-Labeled Viral DNA in the Transfection-Derived Virus Stocks

(Lanes 1, 6, 11 and 16) ^{32}P-labeled DNA prepared from cells infected with passage 28 of the original HSV1 (Patton) series. (Remaining lanes) ^{32}P-labeled DNA from cells infected with the fifth passages of the series generated from the transfections as shown. Table above lanes 2–5: the DNA employed in the transfections. J: HSV1 (Justin) helper DNA. ☆: fragment of defective genome(s) of structure as shown in Figure 2B. ◉: fragment of chimeric defective genome(s) of structure as shown in Figure 2C. O: fragment arising from endogenous defective genome(s). Res: DNA resistant to the restriction enzyme. The fragments Eco RI H; Sal I A, G, S and U; Bam I J, K, N and X; and Kpn I C, D and F correspond to fragments arising from the S component of standard virus DNA. These fragments are seen to be amplified in the transfection-derived virus stocks shown. On the basis of this observation and on the basis of the appearance of large new fragments in the Hind III and Bgl II patterns, the transfection-derived stocks appear to contain mixtures of class I defective genomes arising from the S component sequences, and portions of the adjacent ab sequences.
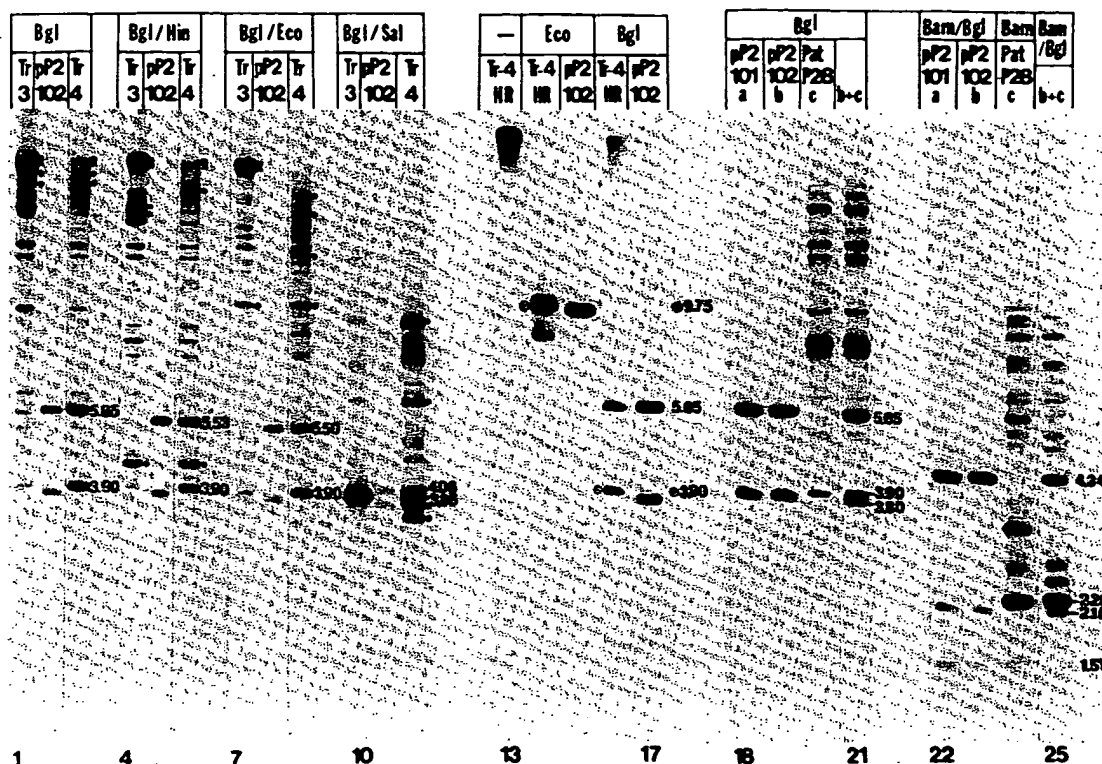
Figure 4. Restriction Enzyme Analyses of Viral DNA in Tr3- and TR4-Derived Virus Stocks and of pP2-101 and pP2-102

(Lanes 1–12) ^{32}P-labeled DNA from cells infected with the fifth passages of the Tr3 and Tr4 series and ^{32}P-labeled pP2-102 DNA were digested with the enzymes shown. R: DNA resistant to the enzyme (approximately 150 kb). ●: fragment generated from endogenous class I defective genome(s) of structure as discussed in the legend to Figure 3. Numbers: sizes, in kilobase pairs, of fragments derived from chimeric defective genomes of structures as described in Figure 2C. Note the deletion (~100 bp) of sequences in the HSV insert of pP2-102, as compared with the corresponding defective genomes in the Tr3- and Tr4-derived virus stocks. (Lanes 13–17) Hpa I-resistant (HR) DNA from cells infected with the fifth passage of the Tr4 series was applied to the gel before (lane 13) or following digestion with the Eco RI (lane 14) or the Bgl II (lane 16) enzymes. (Lanes 15 and 17) ^{32}P-labeled pP2-102 digested with the corresponding enzymes. (Lanes 18–25) Restriction enzyme analyses showing the occurrence of deletions in pP2-101 and pP2-102, as compared with the original defective genome in passage 28 of the Patton series. (Lane 21) A mixture of the Bgl II digests of pP2-102 and passage 28 Patton DNA, to show that the corresponding insert (3.8 kb) does not comigrate with the authentic 3.9 kb Patton repeat unit. (Lane 25) A mixture of pP2-102 and passage 28 Patton DNA digested with the combination of the Bam I and Bgl II enzymes, to show that the 2.26 kb fragment corresponding to Bam I V of standard virus DNA (Locker and Frenkel, 1979b) has been deleted to a 2.16 kb fragment in the pP2-102 clone.

infected with passage 5 of the Tr4 series. This enzyme, which cleaves the helper virus DNA at multiple locations, does not cleave the HSV–pKC7 chimeric repeat units. The Hpa I-resistant DNA, which comigrated in the gels with an uncleaved viral DNA marker (approximately 150 kb), was redigested with the Eco RI and Bgl II enzymes, and we subjected the resultant fragments to electrophoresis on agarose gels along with digests of a recombinant plasmid derived (as described below) by cloning the Bgl II-cleaved 3.9 kb repeat unit into the Bgl II site of pKC7. As seen in Figure 4 (lanes 13–17), the majority of Hpa I-resistant DNA molecules consisted of multiple reiterations of the chimeric HSV–pKC7 seed repeats.

We conclude on the basis of these experiments that ligation of the foreign (pKC7) DNA sequences to the 3.9 kb Patton repeat unit at the Bgl II site results in the introduction of the foreign DNA sequences into full-length (150 kb) defective-virus DNA molecules

that are packaged and successfully propagated during at least five passages of the transfection-derived virus stocks. Because the foreign DNA sequences became amplified within the resultant defective genomes we have termed the HSV-derived vector the HSV amplicon.

The Cloning of the HSV Amplicon
A portion of the 3.9 kb repeat–pKC7 ligation mixture was used to transform bacteria to obtain recombinant plasmids containing the amplicon insert. Resultant clones containing the HSV DNA inserts were all designated by the prefix pP2, to identify the HSV strain (Patton) (P), and the class of HSV defective genomes (class II) (2), from which the amplicon was derived. The structure of a representative cloned recombinant amplicon, pP2-102, is shown in Figure 1. It should be noted that when compared with the original 3.9 kb Patton repeat units (Figure 4), all cloned amplicons

that we have examined contained deletions of small and heterogeneous size (average 100 bp in pP2-102) close to the Kpn I site at position 2.29 (figure 1). This region appeared to be heterogeneous even in populations derived by three sequential isolations of single colonies (R. Spaete and N. Frenkel, manuscript in preparation).

Use of the Cloned Amplicon for the Generation of Chimeric Defective Genomes

The ability of the recombinant plasmid pP2-102 to serve as seed for the generation of full-length, packaged, defective genomes was studied in the following experiment. Duplicate sets of rabbit skin-cell cultures were cotransfected with mixtures of HSV1 (Justin) helper virus DNA and either uncleaved, Bgl II–cleaved or Eco RI–cleaved pP2-102 DNA. Control transfections received the helper virus DNA alone (Tr5 and Tr6), a mixture of helper virus DNA and uncleaved pKC7 DNA (Tr7 and Tr8) or a mixture of helper virus DNA and the recombinant plasmid p29 (Tr9), which is a derivative of pKC7 containing an unrelated HSV DNA insert (Bgl II fragment N, spanning map coordi-

nates 0.62–0.67 of HSV2 (333) DNA). ^{32}P-labeled DNA from cells infected with the third passages of the transfection-derived virus stocks was subjected to restriction enzyme analyses along with ^{32}P-labeled standard HSV1 Justin (P0) DNA. The results of these analyses (Figure 5; additional data not shown) can be summarized as follows.

First, virus derived from the control transfections (Tr5 through Tr9) contained endogenous defective genomes, but no detectable defective genomes, consisting of repeat units corresponding to the added control "seeds."

Second, virus stocks (JP2-102a and JP2-102b) derived from the transfections that received the uncleaved pP2-102 contained chimeric defective genomes consisting of head-to-tail reiterations of the seed cloned amplicon.

Third, virus stocks (JP2-102c and JP2-102d) derived from the transfections that received the Bgl II–cleaved pP2-102 contained high proportions of defective genomes consisting of reiterations of the HSV amplicon insert only. This was expected, because digestion with this enzyme releases the HSV amplicon
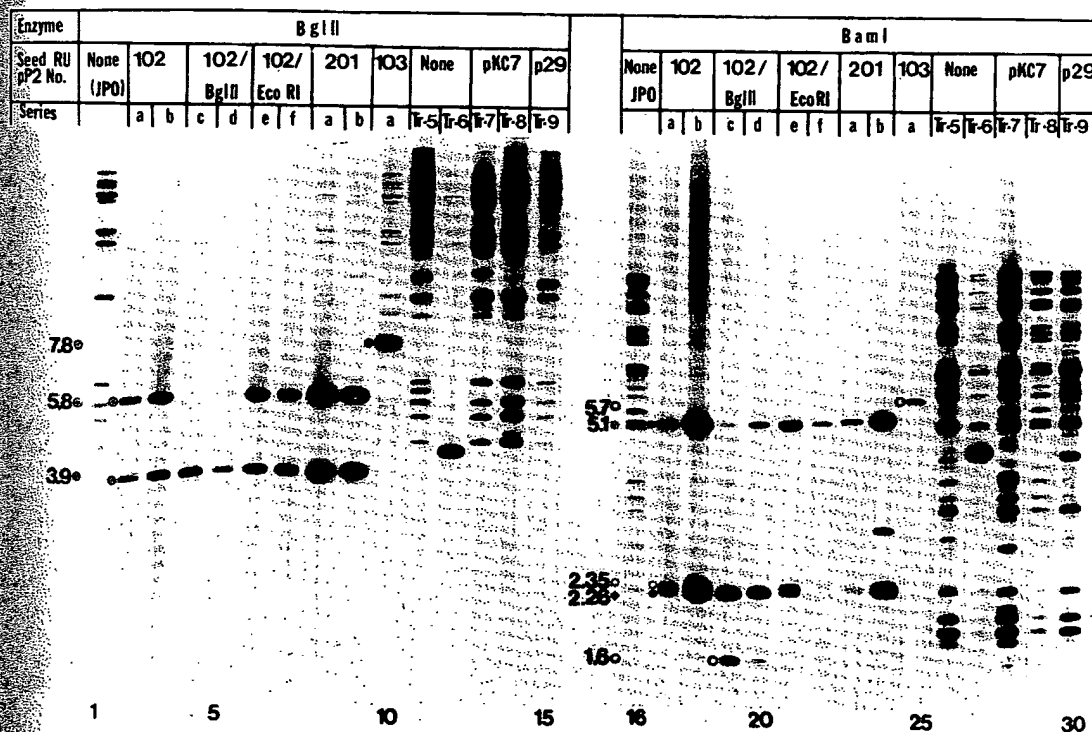

Figure 5. Generation of Defective Genomes from the Recombinant Plasmids pP2-102, pP2-201 and pP2-103

Autoradiographs of gels containing the restriction enzyme fragments of ^{32}P-labeled DNA from cells infected with the third passages of cotransfection-derived series. Each cotransfection received 0.5 μg HSV1 (Justin) helper DNA and 0.10 μg test seed repeat as follows: JP2-102a and JP2-102b (lanes 2, 3, 17 and 18) receiving the uncleaved pP2-102 seed repeats; JP2-102c and JP2-102d (lanes 4, 5, 19 and 20) receiving the Bgl II–cleaved pP2-102; JP2-102e and JP2-102f (lanes 6, 7, 21 and 22) receiving the Eco RI–cleaved pP2-102 seed repeats; JP2-201a and JP2-201b (lanes 8, 9, 23 and 24) receiving the uncleaved pP2-201; JP2-103a (lanes 10 and 25) receiving the uncleaved pP2-103; Tr5 and Tr6 (lanes 11, 12, 26 and 27) containing helper virus DNA only; Tr7 and Tr8 (lanes 13, 14, 28 and 29) receiving pKC7 DNA; Tr9 (lanes 15 and 30) receiving the unrelated p29 recombinant plasmid. (Lanes 1 and 16) ^{32}P-labeled plaque-purified Justin DNA (JP0). Numbers denote sizes, in kilobase pairs, for fragments resulting from cleavage of the regenerated concatemers.

insert from the pP2-102 recombinant plasmid. A minor proportion of the JP2-102c and JP2-102d populations represented concatemers of the pP2-102 repeats, arising most likely from the replication of residual uncleaved seeds or from reunion of the pKC7 and HSV derived segments.

Fourth, transfections containing the added pP2-102 that was linearized by cleavage at the Eco RI site (JP2-102e and JP2-102f) resulted in the generation of chimeric defective genomes of two types: those containing repeat units similar to the pP2-102 (that is, in which the Eco RI site was regenerated), and those in which the sequences immediately flanking the Eco RI site were deleted.

Finally, in all cases, the deletions that were present close to the Kpn I site in the pP2-102 seed were repaired in the generated defective genomes.

We conclude on the basis of this set of studies that in the presence of helper virus DNA, the cloned HSV amplicon pP2-102 could generate full-length and packaged defective-virus genomes.

Generation of a Smaller Derivative Clone of pP2-102

To decrease the size of the cloned amplicon, as well as to facilitate the insertion of additional foreign DNA sequences into the HSV vector, we have derived the recombinant plasmid pP2-103, a Sal I collapse of pP2-102. As shown in Figure 1, this 7.82 kb plasmid contains unique Eco RI, Hind III, Bgl II and Sal I restriction enzyme sites. The ability of pP2-103 to serve as seed repeat units for generation of conca-termic, packaged, defective genomes was tested in a cotransfection experiment similar to that described above, with the HSV1 (Justin) helper virus DNA. As seen in Figure 5 (lane 10), cells infected with passage

3 of the resultant virus stock JP2-103a contained significant proportions of chimeric concatemeric defective genomes.

Transfer of the Cloned Amplicon from Serially Passaged Virus into Bacteria

To test whether monomeric repeat units of the regenerated chimeric defective genomes could be introduced back into bacteria, we prepared DNA from cells infected with the fifth passage of the Tr4 series and digested it with the Eco RI enzyme. The Eco RI-cleaved DNA (containing monomeric repeats) was circularized by ligation and was then used to transform competent Escherichia coli DH1 bacteria (derived by D. Hanahan) to ampicillin resistance. Restriction enzyme analyses of plasmid DNA prepared from a number of the resultant bacterial colonies are shown in Figure 6. These revealed that the rescued plasmids (designated pP2-201a through pP2-201e) consisted of the amplicon fragment inserted in the Bgl II site of pKC7 DNA in an orientation reversed to that of pP2-102. The presence of chimeric repeats containing the amplicon segment in a single orientation most likely reflected the fact that the Tr4-transfected culture itself harbored only a single visible plaque.

The structural features of the pP2-201 clones are summarized in Figure 1. One of these clones was further employed as seed repeat units in two transfections (JP2-201a and JP2-201b) along with helper HSV1 (Justin) DNA. As seen in Figure 5 (lanes 8 and 9) and on the basis of additional restriction enzyme analyses (data not shown), the pP2-201 seed generated concatemeric chimeric defective genomes as expected. This set of experiments demonstrates the dual replication ability (Figure 6) of the pKC7-HSV amplicon recombinant clones in the bacterial and eu-
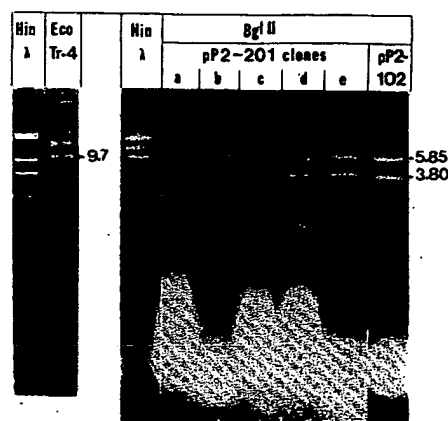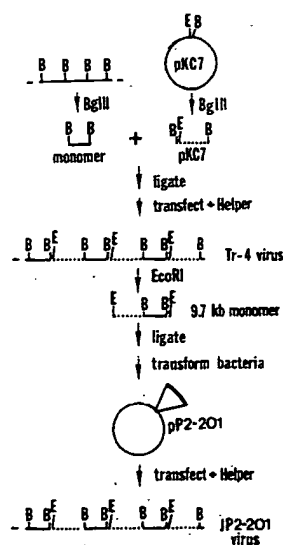

Figure 6. Transfer of the Cloned HSV Amplicon between Bacterial and Animal Cells

(Left) Schematic diagram, showing the generation of the chimeric defective genomes in Tr4; transfer of the monomeric chimeric repeat unit into bacteria by cleavage of the Tr4 DNA with Eco RI; circularization of the resultant monomeric repeats and transformation of bacteria to ampicillin resistance, yielding bacterial colonies containing the pP2-201 plasmids; and the use of pP2-201 as seed repeat units in the generation of chimeric defective genomes in the JP2-201 virus stocks. (Right) Photographs of ethidium-bromide-stained gels. (Lanes Hin λ) 1 μg Hind III-digested λ DNA marker. (Lane Eco Tr-4) Sample of the Eco RI-digested passage 5 Tr4 DNA (1/60 of the DNA extracted from 2 × 10⁶ infected cells) used for the transfer of the chimeric plasmid into bacteria. (Remaining lanes) Bgl II-cleaved plasmid DNAs from a number of the resultant ampicillin-resistant bacterial colonies.

caryotic cell hosts.

Stability of Defective Genomes Containing the Foreign pKC7 DNA

To assess further the stability of the pKC7–HSV defective-virus genomes during virus propagation, we have serially passaged the transfection-derived virus stocks JP2-102b and JP2-201b, and an additional virus stock JP2-102h, which was derived by cotransfection of rabbit skin cells with HSV1 (Justin) helper virus DNA and pP2-102. Each of the passages in the resultant series was generated by use of one fourth of the virus stock constituting the preceding passage as inoculum. An additional series, designated JP2-102b + Helper, was derived in parallel, starting from the third passage of the JP2-102b. Each passage in this series was generated by infection of cell cultures with 1 pfu/cell plaque-purified HSV1 (Justin) in addition to one fourth of the virus stock constituting the preceding passage.

Representative restriction enzyme patterns of ^{32}P-labeled DNA prepared from cells infected with passages 4–12 of the resultant four series are shown in Figure 7. As seen in the figure, the chimeric defective genomes were stable during the serial propagation. In the cases of JP2-102h, JP2-201b and JP2-102b, the serial undiluted passaging was accompanied by cyclic fluctuations in the relative ratios of defective to helper virus DNA, with the most abundant passages containing chimeric genomes in excess of 90%. The chimeric

defective genomes thus exhibited the typical cycling characteristic of defective interfering particles, as described initially by von Magnus (1954) and as later observed for many DNA and RNA viruses (Huang, 1973), including HSV (reviewed by Frenkel, 1981). As seen in Figure 7, the addition of helper virus during the derivation of the JP2-102b + Helper series greatly diminished the fluctuations in the proportion of defective genomes, and all of the resultant virus stocks contained relatively high amounts of chimeric defective genomes. This last observation was consistent with the hypothesis (von Magnus, 1954) that the cycling in the proportion of defective interfering particles through undiluted virus propagation resulted at least in part from the absence of sufficient helper virus necessary to support defective-virus replication.

Discussion

Using bacterial plasmid pKC7 DNA, we have demonstrated that foreign DNA sequences, when linked to the HSV amplicon, can be stably propagated in serially passaged virus populations. Several properties of the HSV amplicon system merit further discussion. First, as previously suggested by us (Locker and Frenkel, 1979a; Frenkel, 1981; Vlazny and Frenkel, 1981; Vlazny et al., 1982) and on the basis of recent additional studies (R. R. Spaete and N. Frenkel, manuscript in preparation) with derivative clones of pP2-102, there are two separate sets of sequences needed
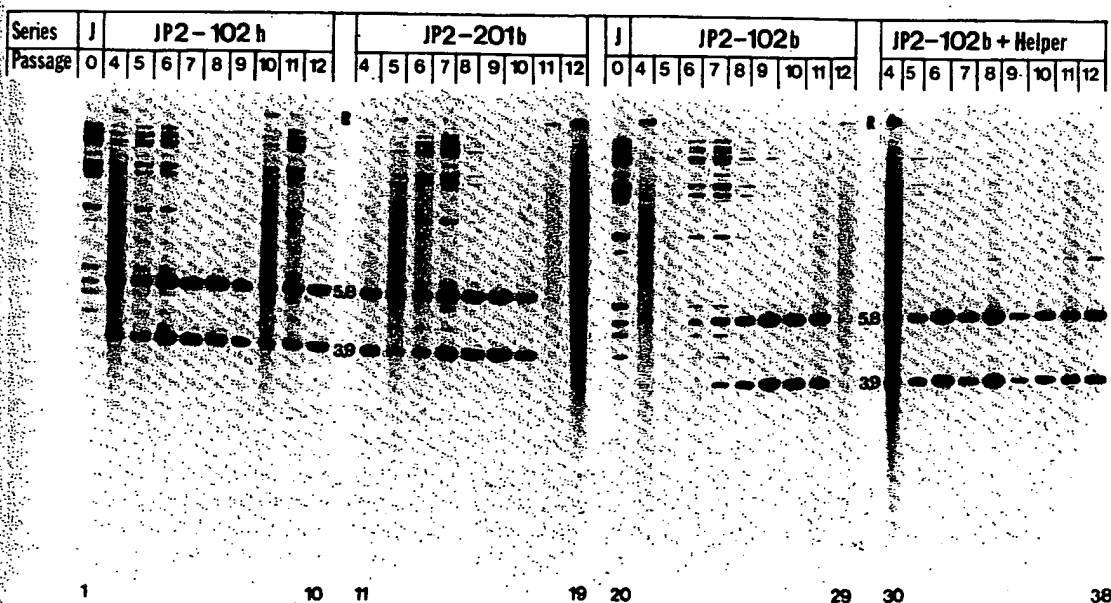

Series	J	JP2–102 h									JP2–201b									J	JP2–102b									JP2–102b + Helper								
Passage	0	4	5	6	7	8	9	10	11	12	4	5	6	7	8	9	10	11	12	0	4	5	6	7	8	9	10	11	12	4	5	6	7	8	9	10	11	12

Figure 7. Stability of the Chimeric Defective Genomes

Autoradiograph of gels containing the electrophoretically separated Bgl II fragments of ^{32}P-labeled DNA from cells infected with plaque-purified HSV1 (Justin) (lanes 1 and 20), or with passages 4–12 of the transfection-derived series shown. Each passage in the JP2-102h, JP2-201b and JP2-102b series was derived by infection of 25 cm² cultures of HEp-2 cells with one fourth of the virus stock constituting the preceding passage. The JP2 passage 4 of the JP2-102b + Helper series was derived by infection of a HEp-2 cell culture with 1 pfu/cell Justin helper virus in addition to one fourth of the virus stock constituting the third passage of the JP2-102b series. Each of the subsequent passages of the JP2-102b + Helper series received 1 pfu/cell helper virus in addition to one fourth of the virus constituting the preceding passage of the series.

for the successful propagation of the class II defective HSV genomes. The first set of sequences corresponds to the terminal portion of the S component of standard virus DNA. We have previously shown that this region contains signal(s) recognized for the cleavage of the HSV DNA concatemers and the packaging of viral DNA into nucleocapsids (Locker and Frenkel, 1979a; Vlazny and Frenkel, 1981; Vlazny et al., 1982). The second set of sequences is located within the U_L region contained in the 3.9 kb repeats of the Patton defective genomes (that is, within map coordinates 0.407–0.429 of standard HSV DNA). This set of sequences is required for the replication of defective-virus DNA and may therefore correspond to a replication origin. It is not clear at present whether the entire origin of replication is present also in the 3.8 kb cloned repeats because, as discussed above, the cloned pP2-102, as well as other cloned derivatives of the Patton repeat units, contain specific deletions that are repaired following the propagation of the chimeric repeats in the infected cells. Thus it is possible that the pP2-102 U_L sequences that appear to be essential for defective-genome propagation correspond to sequences flanking the replication origin. Such sequences will be required to facilitate high-frequency recombination with the corresponding helper virus DNA sequences, resulting in the introduction of a functional origin into the chimeric defective genomes.

Second, in contrast with available virus-vectors derived from papovavirus genomes, the use of the HSV amplicon allows the insertion of relatively large stretches of foreign DNA sequences into chimeric defective genomes. Furthermore, the added DNA sequences are amplified within individual full-sized (approximately 150 kb) DNA molecules. Current data strongly suggest that this amplification results from a rolling circle replication of monomeric repeats, resulting in the generation of homopolymers even in virus populations containing mixtures of different-sized repeat units (Becker et al., 1978; Locker and Frenkel, 1979a; Vlazny and Frenkel, 1981).

Third, the chimeric pKC7–HSV genomes that we have studied appeared to persist through undiluted virus propagation in the absence of selective pressure. This apparent stability must reflect as yet unknown parameters in the dynamics of replication and propagation of defective HSV genomes. Specifically, data from previous studies of serially passaged virus populations strongly suggested that different-sized repeat units within a given HSV series arose by deletions from a single defective-genome "progenitor" repeat. Thus similar modifications in defective-genome sequences (as compared with the parental helper virus DNA) were found to be present in different-sized repeat units within certain serially passaged virus populations (derived from HSV2(G) and HSV1

tsLB2 (HFEM); Frenkel, 1981; Locker et al., 1982). However, the generation of different-sized repeats appeared to be restricted to early stages of the serial propagation because no new species of defective genomes became amplified during further prolonged propagation (from the third to the 40th passage) of these series. Thus additional studies will be required to assess firmly the stability of defective chimeric genomes, in particular those designed to contain large inserts of foreign DNA sequences.

Finally, virus populations containing the chimeric defective genomes can be used to infect a wide variety of host-cell species that are susceptible to HSV. In this respect the HSV amplicon differs from the novel and most useful SV40-derived vector system developed recently by Gluzman (1981; see, for example, Mellon et al., 1981). This latter system requires the use of the SV40-transformed COS monkey cells to supply in trans the T function needed for the replication of the transfecting SV40-vector-linked foreign DNA sequences.

When compared with gene cotransfer systems utilizing selectable enzyme markers (see, for example, Wigler et al., 1979; Mulligan and Berg, 1980), the use of the packaged HSV chimeric defective genomes enables the efficient and synchronous introduction of foreign DNA sequences into the majority of the manipulated cells. At the same time, exposure of cells to virus populations containing mixtures of defective and standard lytic viruses ultimately results in cell death. Whereas the selectable enzyme approaches are generally suitable for studies of long-term association of the foreign DNA within the recipient cells, the HSV amplicon system provides an efficient means to obtain a short-term presence of foreign DNA sequences in recipient cells albeit coupled with the presence of the lytic helper viruses. Alternatively, concatemeric DNA containing amplified foreign genes may be efficiently introduced into eucaryotic cells via dilute infections designed to minimize the presence of helper virus in the manipulated cells.

Previous studies of the expression of serially passaged virus populations containing class I or class II defective-virus genomes have shown that viral genes present within defective-genome repeat units are most efficiently expressed (reviewed by Frenkel, 1981). Thus the HSV amplicon can be used in studies requiring abundant expression of viral genes. In contrast, it is unknown at this stage whether multiple copies of intact cellular genes will indeed be expressed in the HSV amplicon system, because infections of cells with wild-type HSV are generally accompanied by the shut off of host polypeptide synthesis. We have recently derived HSV mutants that are defective with respect to their ability to shut off host polypeptide synthesis (G. S. Read and N. Frenkel, manuscript submitted). We are currently testing whether such mutants could

serve as useful helper viruses in studies designed to obtain expression of cellular genes from within chimeric defective genomes.

Experimental Procedures

Cells and Viruses

Human epidermoid 2 (HEp-2) cells and African Green monkey kidney (Vero) cells were obtained from Flow Laboratories). Rabbit skin cells were obtained from B. Roizman. The HSV1 strain Justin was obtained from A. Sabin, and the HSV2 strain 333 from F. Rapp. The serially passaged HSV1 (Patton) was obtained from B. K. Murray at passage 21 and was further propagated in HEp-2 cells to passage 28 at 1:4 dilutions.

Preparation of Purified Viral DNA, Restriction Enzyme Analyses and Elution of DNA from Gels

Viral DNA to be used in transfections or in bacterial cloning was prepared as previously described by Locker and Frenkel (1979b). Restriction enzymes were purchased from New England Bio-Labs and from Bethesda Research Laboratories. Electrophoresis of restriction enzyme digests for autoradiography was carried out in 0.5%–1% agarose gels (Bethesda Research Laboratories). For the preparation of ^{32}P-labeled Hpa I-resistant DNA to be redigested with additional restriction enzymes, the electrophoresis following the Hpa I digest was done in 0.5% low-melting-point agarose (Bethesda Research Laboratories). The gel was stained with ethidium bromide, and slices containing the enzyme-resistant DNA were excised, melted at 68°C, mixed with the appropriate restriction enzyme reagents and incubated with an excess of the second restriction enzyme for 2–4 hr. For the preparation of the 3.9 kb repeat units to be used in cloning and in transfection protocols, the Bgl II digest of passage 28 Patton viral DNA was electrophoresed in 0.5% low-melting-point agarose. Following ethidium bromide staining, gel slices containing the 3.9 kb repeats were melted at 68°C, cooled to 37°C, brought to a concentration of 0.1 M NaCl and extracted twice with phenol (equilibrated with 0.05 M Tris-HCl [pH 8.0], 0.1 M NaCl) at 37°C. The resultant aqueous phase was then concentrated with n-butanol, extracted twice with ether and dialyzed extensively against TE (0.01 M Tris-HCl [pH 8.0], 0.001 M EDTA [pH 7.0]). The dialyzed DNA was then precipitated with ethanol.

Preparation of Plasmid DNA and Construction of Recombinant Plasmids

Large-scale preparations of plasmid DNA were made by the standard cleared lysate procedure and centrifugation in cesium chloride–ethidium bromide gradients as described by Clewell and Helinski (1970). For the preparation of ^{32}P-labeled plasmid DNA, the bacteria were grown in broth containing 0.02 mCi/ml ^{32}P-orthophosphate. Plasmid DNA was prepared by the rapid boiling method described by Holmes and Quigley (1981), treated with RNAase A (50 µg/ml) at 37°C for 15 min and chromatographed on Sephadex G-50 columns to remove oligoribonucleotides. For the cloning of the 3.9 kb Patton repeat unit, Bgl II-cleaved pKC7 DNA (Rao and Rogers, 1979) was treated with calf alkaline phosphatase (Boehringer-Mannheim), and was ligated to the gel-extracted 3.9 kb repeat unit DNA. E. coli DH1 (gift from D. Hanahan) was transformed with the ligated DNA by the standard calcium shock method (Mandel and Higa, 1970). Plasmids from ampicillin-resistant, kanamycin-sensitive colonies were prepared by the rapid boiling method as described above and analyzed with restriction enzymes.

Transfection of Cells, and Propagation and Analyses of Transfection-Derived Series

Approximately 0.5 µg intact helper virus DNA was introduced into rabbit skin cells (25 cm² cultures) along with 0.05–0.15 µg test seed repeat units and 5 µg calf thymus DNA carrier. Transfection was done by the calcium phosphate precipitation, as described by Ruyechan et al. (1979). Virus stocks derived from cotransfections that received

helper virus and recombinant plasmid amplicons were designated by the first letter of the helper virus strain followed by the amplicon plasmid designation. Virus stocks derived from transfections receiving helper virus DNA alone or with uncloned amplicon or with unrelated recombinant plasmids were designated by the prefix Tr. All the transfection-derived virus stocks were serially propagated in HEp-2 cells (25 cm² cultures) at 1:4 dilution. For the preparation of ^{32}P-labeled serially passaged virus DNA, 25 cm² infected Vero cell cultures were labeled with 0.012 mCi/ml ^{32}P-orthophosphate 6–24 hr after infection. Cell lysates were prepared by SDS–proteinase K digestion as described by Locker and Frenkel (1979b). Following addition of 2 volumes of isopropanol, the infected-cell DNA was spooled on a glass rod, dried, dissolved in TE and analyzed with restriction enzymes.

Transfer of the Amplicon from Infected Cells into Bacteria

Total infected-cell DNA was prepared from 25 cm² Vero cell cultures infected with passage 5 of the Tr4 virus series. One microgram of this DNA was digested with the Eco RI enzyme, heated to 65°C for 10 min and ligated in a total volume of 300 µl, to obtain circularization of DNA fragments (Dugaiczyk et al., 1975). An aliquot of the ligation mixture was used to transform competent DH1 bacteria. Ampicillin-resistant colonies were grown up, and their plasmids were analyzed with restriction enzymes.

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